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REQUEST FOR GRANT OF A PATENT

9024503.6

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION

I	Applicant's or Agent's reference (Please insert if available)	COB/6013379	
II	Title of invention	BINDING SUBSTANCES	
III	Applicant or Applicants (See note 2)		
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	Country	State	
	Address		
IV	Inventor (see note 3)	<p>(a) The applicant(s) is/are the sole joint inventor(s)</p> <p>or</p> <p>(b) A statement on Patents Form No. 7/77 is/will be furnished</p>	
V	Name of Agent (if any) (See note 4)	MEWBURN ELLIS	
		ADP CODE NO 109001	
VI	Address for Service (See note 5)	2 Cursitor Street London EC4A 1BQ	
VII	Declaration of Priority (See note 6)		
	Country	Filing date	File number
VIII	The Application claims an earlier date under Section 8(3), 12(6), 15(4), or 37(4) (See note 7)		
	Earlier application or patent number	and filing date	

IX Check List (To be filled in by applicant or agent)

A The application contains the following number of sheet(s)

1 Request 1 Sheet(s) 1 Priority document
2 Description 46 Sheet(s) Translation of priority document
3. Claim(s) Sheet(s) 3. Request for Search.....
4 Drawing(s) 19 Sheet(s) 4 Statement of Inventorship and
Right to Grant.....
5 Abstract Sheet(s)

B The application as filed is accompanied by:-

X It is suggested that Figure No..... of the drawings (if any) should accompany the abstract when published.

XI Signature (See note 8) *Hewburn Ellis*

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BINDING SUBSTANCES

The present invention relates to binding substances. The present invention also relates to methods for the production of binding substances eg binding molecules and to the biological binding molecules produced by these methods. The present invention also relates to: a) the production of antibodies, receptor molecules and fragments and derivatives of these antibodies and receptor molecules; b) viruses encoding the above identified molecules, which viruses have the ability to present said molecules at their surfaces; c) packages comprising a virus and an above identified molecule presented at the viral surface; and d) screening techniques utilising the unique properties of these packages.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 *Nature* 256: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because the antibody-secreting mammalian cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). Each chain has a constant region (C) and a variable region (V). The antibody has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ

from one antibody to another, and which together are responsible for recognising the antigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Binding fragments are the F_V fragment which comprises the V_L and V_H of a single arm of the antibody, and the dAb fragment (Ward, E.S. et al., *Nature* 341, 544-546 (1989); which consists of a single heavy chain variable domain (V_H).

Although the F_V fragment is coded for by separate genes, it has proved possible to construct a linker that enables them to be made as a single protein chain (known as single chain F_V (scFv); Bird, R.E. et al., *Science* 423, 423-426 (1988) Huston, J.S. et al., *Proc. Natl. Acad. Sci., USA* 85, 5879-5883 (1988)) by recombinant methods.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (*Clinical Applications of Monoclonal Antibodies*. Edited by E. S. Lennox. *British Medical Bulletin* 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 µg/ml). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 µg/ml). However, the repeated administration of these foreign rodent proteins to humans can lead to harmful hypersensitivity reactions. In the main therefore, these

rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different antibody producing cells with different specificities, can be sampled, compared to how many need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. 239; 1-16, (1990)). For example, the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.

This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal is immunised with an immunogen expressing that epitope. The animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure. However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed) such an approach is not practically or ethically feasible.

In the last few years, these problems have in part,

been addressed by the application of recombinant DNA methods to the isolation and production of antigen binding fragments of an antibody molecule in bacteria such as E.coli. Furthermore, the use of polymerase chain reaction (PCR) 5 amplification (Saiki, R.K., et al., *Science* 239, 4387-491 (1988)) to isolate antibody producing sequences from cells and organs, has great potential for speeding up the timescale under which specificities can be isolated. Amplified V_H and V_L genes are cloned directly into vectors 10 for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, *Proc. Natl. Acad. Sci., USA* 86, 3833-3837; Ward, E.S., et al., 1989 *supra*; Larrick, J.W., et al., 1989, *Biochem. Biophys. Res. Commun.* 160, 1250-1255; Sastry, L. et al., 1989, *Proc. Natl. Acad. Sci., USA*, 86, 5728-5732). 15 Conversely, some of these techniques can exacerbate the screening problems. For example, large separate heavy and light chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, *Science* 246, 1275- 20 1281). Crucially however, the information held within each cell, namely the specific combination of one light chain with one heavy chain, is lost. This loses most, if not all, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single 25 heavy chain variable domains (dAbs; Ward, E.S., et al., 1989, *supra*) do not suffer this drawback, but because not all antibody heavy chain variable regions are capable of binding antigen, more have to be screened.

In addition, the problem of directly screening many 30 different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameliorates or overcome one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg of the order of 10^6 and 35 higher) rapid sorting at each cloning round, and rapid

transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and retain antibody on their surface. It has already been shown that antibody fragments can be secreted through bacterial membranes with the appropriate signal peptide (Skerra, A., and Pluckthun, A., 1988, Science 240, 1038-1040; Better, M. et al., 1988, Science 240, 1041-1043). However, it has not been shown how an antibody or antibody fragment can be held on the bacterial cell surface in a configuration which allows efficient sampling of its antigen binding properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position.

Bacteriophage make attractive candidates because in general their surface is a much simpler structure, they can be grown easily in large numbers, are amenable to the practical handling involved in many potential mass screening programmes and they carry genetic information for their own synthesis within a small, simple package. The difficulty has been to practically solve the problem of how to use bacteriophages in this manner. For example, a Genex Corporation patent application number PCT/US88/00716 has proposed that the bacteriophage lambda would be a suitable vehicle for the expression of antibody molecules, but no proposals provide a teaching which enables the general idea to be carried out. For example PCT/US88/00716 does not demonstrate that any sequences: a) have been expressed as a fusion with gene V; b) have been expressed on the surface of lambda; and c) retain biological activity. Furthermore there is no teaching on how to screen for suitable fusions.

The problem of how to use bacteriophages is in fact a

difficult one. The antibody molecule must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the antibody itself should be biologically active. Thus the antibody should fold 5 efficiently and correctly and be presented for antigen binding. However, solving the problem for antibody molecules and fragments would also provide a general method for the screening of many receptor molecules.

Surprisingly, the applicants have been able to 10 construct a bacteriophage that expresses and presents on its surface large binding molecules (eg large biologically functional antibody molecules) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule presented 15 at the viral surface a 'package'. Where the binding molecule is an antibody (or a fragment or derivative of an antibody), the applicants call the package a phage antibody. However, except where the context demands otherwise, where the term phage antibody is used generally it should also be 20 interpreted as referring to any package comprising a virus particle and a binding molecule presented at the viral surface. Phage-antibodies (pAbs) are likely to find a range of applications in screening antibody V-genes encoding antigen binding activities. For example, pAbs could be used 25 in cloning and rescue of hybridomas (Orlandi, R., et al (1989) PNAS 86 p3833-3837), and in the screening of large combinatorial libraries (such as found in Huse, W.D. et al., 1989, Science 246, 1275-1281) In particular, rounds of selection using pAbs may help in rescuing the higher 30 affinity antibodies from the latter libraries. It may be preferable to screen small libraries derived from antigen-selected cells (Casali, P., et al., (1986) Science 234 p476-479) to rescue the original VH/VL pairs comprising the V region of an antibody. The use of pAbs may also allow the 35 construction of entirely synthetic antibodies. For example, V-gene repertoires could be made in vitro by combining unarranged V genes, with D and J segments. Libraries of

pAbs could then be selected by binding to antigen, hypermutated in the antigen-binding loops in vitro and subjected to further rounds of selection and mutagenesis.

The demonstration that a functional antigen-binding site can be expressed on the surface of phage, has implications beyond the construction of novel antibodies. For example, if other protein domains can be expressed at the surface of a phage, phage vectors could be used to clone and select genes by the binding properties of the expressed protein. Furthermore, endless variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. In effect other protein architectures might serve as "nouvelle" antibodies. One class of molecules that could be useful in this type of application are receptors. For example, a specific receptor could be expressed on the surface of the phage such that it would bind its ligand. The receptor could then be modified by, for example, in vitro mutagenesis and variants having higher binding affinity for the ligand selected. The screening may be carried out according to one or more of the formats described below with reference to figure 2. Figure 2 refers particularly to phage antibodies. In the situation discussed above the phage antibody is replaced with a phage receptor and the antigen with a ligand 1.

Alternatively, the receptor-phage could be used as the basis of a rapid screening system for the binding of ligands, altered ligands, or potential drug candidates. The advantages of this system of simple cloning, convenient expression, standard reagents and easy handling makes the drug screening application particularly attractive. In the context of this discussion, receptor means a molecule that binds a specific, or group of specific, ligand(s); the natural receptor could be expressed on the surface of a population of cells, it could be the extracellular domain of such a molecule (whether such a form exists naturally or not), or it could be a soluble molecule performing a natural

binding function in the plasma or within a cell or organ.

Although throughout this application the applicant discuss the possibility of screening for higher affinity variants of phage-antibodies, they recognise that in some 5 applications, for example low affinity chromatography (Ohlson, S. et al Anal. Biochem. 169, p204-208 (1988)), it may be desirable to isolate lower affinity variants.

The system of expressing binding molecules on the phage surface could also be used as a primary cloning system. For 10 example, a cDNA library could be constructed and inserted into the bacteriophage and this library screened for the ability to bind a ligand. The ligand/binding molecule combination could include receptor/ligand, enzyme/substrate (or analogue), nucleic acid binding protein/nucleic acid 15 etc. This could be a preferred method to isolate a clone of either molecule in the pair, if the other molecule was available.

Targeted gene transfer

20 A useful and novel set of applications makes use of the binding protein on the phage to target the phage genome to a particular cell or group of cells. For example, a phage-antibody specific for a cell surface receptor could be used 25 to bind to the target cell surface. The phage could then be internalised, either through the action of the receptor itself or as the result of another event (eg. an electrical discharge such as in the technique of electroporation). The phage genome would then be expressed if the relevant control 30 signals (for transcription and translation and possibly replication) were present. This would be particularly useful if the genome contained a sequence whose expression was desired in the target cell (along with the appropriate expression control sequences). A useful sequence might 35 confer antibiotic resistance to the recipient cell or label the cell by the expression of its product (eg. if the sequence expressed a detectable gene product such as a

luciferase, see White, M, et al, Techniques 2(4), p194-201 (1990)), or confer a particular property on the target cell (eg. if the target cell was a tumour cell and the new sequence directed the expression of a tumour suppressing 5 gene), or express an antisense construct designed to turn off a gene or set of genes in the target cell, or express a gene or gene product designed to be toxic to the target cell.

This technique of "targeted gene transfer" has a number 10 of uses in research and also in therapy and diagnostics. For example, gene therapy often aims to target the replacement gene to a specific cell type that is deficient in its activity; targeted pAbs provide an answer to this problem. In diagnostics, phage specific for particular 15 bacteria or groups of bacteria have been used to target marker genes, eg. luciferase, to the bacterial host (see, for example, Ulitzer, S., and Kuhn, J., EPA 85303913.9). If the host range of the phage is appropriate, only those bacteria that are being tested for, will be infected by the 20 phage, express the luciferase gene and be detected by the light they emit. This system has been used to detect the presence of *Salmonella*. One major problem with this approach is the initial isolation of a bacteriophage with the correct host range and then cloning a luciferase gene 25 cassette into that phage, such that it is functional. The pAb system allows the luciferase cassette to be cloned into a well characterised system (filamentous phage) and allows simple selection of an appropriate host range, by modifying the antibody (or other binding molecule) specificity the pAb 30 contains.

The applicant has also shown that enzymes can be expressed on the phage surface. Useful applications of this invention include the cloning of enzyme coding genes, or the design and selection of mutant enzymes with enhanced 35 properties on particular substrates. For example, conditions can be used whereby the enzyme (or modified enzyme) binds a particular substrate, product or

intermediate (or analogues of them) to identify phage from a library containing a desired activity or by subjecting phage already expressing the enzyme, to in vitro mutagenesis, followed by selection of those variants with a desired level 5 of binding and/or catalysis.

The present applicants have also been able to develop novel screening systems and assay formats which depend on the unique properties of these packages eg phage antibodies.

The present invention provides a method for producing a 10 package which method comprises the steps of:

- a) inserting a nucleotide sequence encoding the binding molecule within a viral genome;
- b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed by 15 the virus presented at its surface.

The present invention also provides a method for producing a binding molecule specific for a particular epitope which comprises producing a package as described above and the additional step of screening for said binding 20 molecule by binding of said molecule to said epitope. The method may comprise one or more of the additional steps of: i) separating the package from the epitope; ii) recovering said package; and iii) using the inserted nucleotide sequence in a recombinant system to produce the binding 25 molecule separate from virus. The screening step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus.

30 In the above methods, the binding molecule may be an antibody, or a fragment or derivative of an antibody. Alternatively, the binding molecule may be an enzyme or receptor and fragments/derivatives of any such enzymes or receptors.

35 In the above methods, the virus may be a filamentous F-specific bacteriophage. The filamentous F-specific bacteriophage may be fd. In particular, it may be a

tetracycline resistant version of fd known as fd-tet. The nucleotide sequence may be inserted within the gene III region of fd. The sequence may be inserted after the signal sequence of gene III, preferably after amino acid+1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the DNA to be inserted. For example, the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the V_H domain, or QVQLQ and LEIKR which occur at either end of the F_v (combined $V_H + V_L$) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown in figure 4(2) B and C as described above, may be used to flank the insertion site for any DNA to be inserted, whether or not that DNA codes an immunoglobulin.

In the above methods the nucleotide sequences inserted within the viral genome may be derived from eg mammalian spleen cells or peripheral blood lymphocytes. The mammal may be immunised or non-immunised. Alternatively, the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence. The phage particle presenting said binding molecule may remain intact and infectious.

As previously mentioned, the present invention also provides novel screening systems and assay formats. In these systems and formats the gene sequence encoding the binding molecule (eg the antibody) of desired specificity is separated from the general population having a range of specificities by the fact of its binding to a specific target (eg the antigen or epitope).

Thus, the present invention provides a method of screening a population of phage antibodies (where the binding molecule is an antibody) for a phage antibody with a desired specificity, which comprises contacting said population of phage antibodies with a desired epitope and separating phage antibody which binds to said epitope, from said epitope. The means for separating any binding phage antibodies may be varied in order to obtain binding phage antibodies with different binding affinities for said epitope.

Alternatively, in order to obtain high affinity phage antibodies the epitope may be presented to the population of phage antibodies already with a binding member for said epitope bound thereto, in which case, phage antibodies with a higher binding affinity for said epitope than said bound binding member will displace said bound binding member. The high affinity phage antibodies can then be separated from said epitope.

Separation of phage antibodies from said epitope may be achieved by eg elution techniques well known in the art, infection of suitable bacteria etc.

The present invention also provides packages as defined above and binding molecules (eg antibodies, enzymes, receptors; fragments and derivatives thereof), obtainable by use of any of the above defined methods, systems and formats.

The applicants have chosen the filamentous F- specific bacteriophages as an example of the type of phage that could provide a vehicle for the expression of antibodies and antibody fragments and derivatives on their surface and facilitate subsequent screening and manipulation.

The F-specific phages (eg f1, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980). The single stranded DNA genome (approximately 6.4 Kb) is extruded through the bacterial

membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1 μ m in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four molecules of the adsorption molecule encoded by viral gene III. The latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978, in The Single Stranded DNA Phages, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 *supra*.) This places some restraint on their use. The applicants have recognized that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. The protein itself is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, *Virology* 167: 156-165). Furthermore, it is possible to insert some foreign sequences (with no biological function) into various positions within this gene (Smith, G., 1985 *Science* 228: 1315-1317., Parmley, S.F. and Smith, G.P *Gene*: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, *J. Biol. Chem.*, 263: 4318-4322). In these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 *Virology* 39:42-53., Grant, R.A., et al., 1981, *J. Biol. Chem.* 256: 539-546 and Armstrong, J., et al., *FEBS Lett.* 135: 167-172 1981.) including: i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; ii) a domain that anchors the mature protein into the bacterial cell membrane (and also the phage coat); and iii) a domain that specifically binds to the phage receptor the F-pilus of the host bacterium. Short sequences derived from protein molecules have been

inserted into two places within the mature molecule (Smith, G., 1985 *supra.*, and Parmley, S.F. and Smith, G.P., 1988 *supra.*) into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the 5 N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of specific antisera, the peptides inserted into this position were shown to be on the surface of the phage. These authors 10 were also able to purify the phage using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

15 Retaining the biological function of a molecule when it is expressed in a radically different context to its natural state is difficult. The demands on the structure of the molecule are heavy. In contrast, retaining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the structure of the molecule. For example, it is the rule rather than the 20 exception that polyclonal antisera will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a region 25 that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding biological function.

30 This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a molecule, than does the retention of a biological function.

35 The applicants have investigated the possibility of inserting biologically active antibody fragments into the gene III region of fd to create a large fusion protein. As

is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to retain antigen-binding; and most of the functions of gene III must be retained. The applicants' approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. The initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene 9, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for several reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links V_H - V_L to C_H1 - C_L (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that expresses on its surface large biologically functional antibody molecules and which remains intact and infectious. Furthermore, the phages bearing antibodies of the correct specificity, can be selected from a background where the majority of phages do not show this specificity.

The population of antibody molecules inserted into the phage can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orlandi,

R., et al., 1989 supra; Lerrick, J.W., et al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques 7, p. 360-366; Ward, E.S., et al., 1989 supra; Sastry, L., et al., 1989 supra.) Each individual phage antibody in the 5 resulting library of phage antibodies will express antibody derived fragments that are monoclonal with respect to its antigen-binding characteristics.

The disclosure made by the present applicants is important and provides a significant breakthrough in the 10 technology relating to the production of biological binding molecules, their fragments and derivatives by the use of recombinant methods.

In standard recombinant techniques for the production of antibodies, an expression vector containing sequences 15 coding for the antibody polypeptide chains is used to transform eg E.coli. The antibody polypeptides are expressed and detected by use of standard screening systems. When the screen detects an antibody polypeptide of the desired specificity, one has to return to the particular 20 transformed E.coli expressing the desired antibody polypeptide. Furthermore, the vector containing the coding sequence for the desired antibody polypeptide then has to be isolated for use from E.coli in the further processing steps.

25 In the present invention however, the desired antibody polypeptide when expressed, is already packaged with its gene coding sequence. This means that when the screen detects an antibody polypeptide of desired specificity, there is no need to return to the original culture for 30 isolation of that sequence.

Because the phage antibody is a novel structure that contains an antibody of monoclonal antigen-binding specificity on the surface of a relatively simple structure also containing the genetic information encoding its 35 function, phage antibodies that bind antigen can be recovered very efficiently by either eluting off (eg using diethylamine, high salt etc) and infecting suitable

bacterial or by denaturing the structure and specifically amplifying the antibody encoding sequences using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the phage antibody.

5 Individual phage antibodies expressing the desired antigen-binding specificity can be isolated from the complex library using the conventional screening techniques (eg as described in Harlow, E., and Lane, D., 1988, *supra*). One example is illustrated in figure 2(i). This shows antigen (ag) bound to a solid surface (s). The population of phage antibodies is then passed over the antigen, and those individuals p that bind are retained after washing, and optionally detected with detection system d. One possible detection system based upon anti-fd antisera is illustrated
10 below in example 4. Since the bound phage antibody can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional techniques.
15

20 The efficiency of this screening procedure for phage antibodies and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique
25 allows the rapid isolation of antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible once a complete library of the immune
30 repertoire has been constructed.

Affinity Maturation Screening Formats

35 The applicants have also devised a series of novel screening techniques that are practicable only because of the unique properties of phage antibodies. The general outline of some screening procedures is illustrated in figure 2.

The population/library of phage antibodies to be

screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phageantibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis (Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can be screened in one or more of the formats described below with reference to figure 2, to derive those individual phage antibodies whose antigen binding properties are different from sample c. Examples of the possible screening formats are:

Binding/Elution

Referring to figure 2(i) population p binds to antigen ag fixed to a solid support s. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant phage antibody (or a set of unrelated phage antibody) p is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical

industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotype antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

In some instances it may prove advantageous to pre-select population p . For example, in the anti-idiotype example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a phage antibody, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c . This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound $p+c$ are eluted from the antigen and used to infect bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

The novel structure of the phage antibody molecule can be used in a number of other applications some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, phage antibodies combine the ability to bind the specific antigen with the amplification, if the major coat protein is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used,

for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the phage antibody can be used as a marker particularly with respect to physical methods of detection such as electron microscopy and/or some biosensors, eg. surface plasmon resonance.

Diagnostic Assays

The phage antibody molecule also has advantageous uses in diagnostic assays, particularly where separation can be effected using its physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simplest antibody molecule IgG.

Figure 2 shows schematically novel screening techniques which utilise the unique properties of phage antibodies.

Figure 3 shows a scheme for the construction of vectors.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the oligonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, oligonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. these sequences are drawn in the sense orientation with respect to gene III; a) fd-tet (and

FDTdBst) b) FDTPs/Bs and c) FDTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 *supra*.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the sequence of the anti-lysozyme single chain Fv and surrounding sequences in scFvD1.3 myc showing the N-terminal pel B signal peptide sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, *supra*.). Also highlighted is the peptide sequence linking the V_H and V_L regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane, D., 1988 *supra*.

Figure 6 shows the effect of varying the amount of supernatant on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM NaHCO₃.

Figure 7 shows the effect of varying the coating concentration on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated with the specified concentration of either BSA or lysozyme.

Figure 8 shows the sequence around the cloning site in gene III of fd-Cat2.

Figure 9 shows the binding of pAb (D1.3) to lysozymes. Binding of phage as detected by ELISA to (a) hen egg-white lysozyme (HEL) (b) turkey egg-white lysozyme (TEL), (c) human lysozyme (HUL), (d) bovine serum albumin (BSA). A further control of (c) fd-CAT1 to HEL.

Figure 10 shows a map of fab D1.3 in pUC19.

Figure 11 shows the ELISA results providing a comparison of lysozyme-binding by phage-Fab and phage-ScFv. Vector=fd-CAT2 (example 5); fdSCFV(OX)=pAbNQ11 (Example 9); fdVHCH1(D1.3)=grown in normal cells (ie. no light chain, see

example 7); fd fab-fdVHCH1 (D1.3) grown in cells containing D1.3 light chain; fdSCFV(D1.3)=pAbD1.3.

Figure 12 shows oligonucleotide probing of affinity purified phage. 10^{12} phage in the ratio 1 pAb(D1.3) in 4×10^4 fd-CAT1 phages were affinity purified and probed with an oligonucleotide specific for pAb (D1.3) (a) filter after one round of affinity purification (900 colonies total) and, (b) after two rounds (372 colonies total).

Figure 13 shows the sequence of the anti-oxazalone antibody NQ11.

Figure 14 shows the ELISA results for binding of pAb NQ11 and pAb D1.3 and Vector FDTPs/xh to specified antigens.

Figure 15 shows the sequence surrounding phoA insertion in fd-phoA1. The restriction sites used for cloning are shown, as well as the amino acids encoded by phoA around the insertion site. The first five amino acids of the mature fusion come from gene 3.

Figure 16(1) shows the structure of gene 3 and figure 16(2) shows the peptide linker sites A and B.

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989, *supra*: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides, filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N et al., 1980, *supra*.) was obtained from the American Type Culture Collection (ATCC

No. 37000) and transformed into competent TG1 cells (genotype: K12 δ (lac-pro), sup E, thi, hsdD5/F'traD36, pro A+B+, Lac 1 q , lac 6M15).

Viral particles were prepared by growing TG1 cells containing the desired construct in 10 to 100 mls 2xTY medium with 15 μ g/ml tetracycline for 16-24 hours. The culture supernatant was collected by centrifugation for 10 mins at 10,000 rpm in an 8 x 50 ml rotor, Sorval RC-5B centrifuge. Phage particles were precipitated by adding 1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and leaving at 4°C for 1 hour. These were spun for 15 minutes as described above and the pellets resuspended in 10 mM Tris/HCl pH 8, 1 mM EDTA to 1/100th of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 minutes in a microcentrifuge. Single stranded DNA for mutagenesis or sequencing was prepared from concentrated phage according to Sambrook, J., et al., 1989, *supra*.

Example 1

Design of Insertion Point Linkers and Construction of Vectors

The vector fd-tet has two BstEII restriction sites flanking the tetracycline resistance gene (fig 3). Since the strategy for inserting the V_H fragments was to ligate them into a newly inserted BstEII site within gene III, it was advantageous to delete the original BstEII sites from fd-tet. This was achieved by digesting fd-tet with the restriction enzyme BstEII, filling-in the 5' overhangs and re-ligating to generate the vector FDT δ Bst. Digestion of fd-tet with BstEII (0.5 units/ μ l) was carried out in 1x KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (PH 7.5), 10 mM magnesium acetate, 50 μ g/ml bovine serum albumin, 0.5 mM (Sambrook, J., et al., 1989, *supra*.) with DNA at a concentration of 25 ng/ μ l. The 5' overhang was filled in, using 2x KGB buffer, 250 μ M each dNTP's

(Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/ μ l. After incubating for 1 hour at room temperature, DNA was extracted with phenol/chloroform and precipitated with ethanol.

Ligations were carried out at a DNA concentration of 50ng/ μ l for 1 hour at room temperature using T4 DNA ligase (40 units/ μ l). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 μ g/ml tetracycline. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 μ g/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified from the resulting clones using the gene-clean II kit (Biol01 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme Clal. A clone was chosen which gave the same Clal pattern as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of FDT8Bst was used to generate vectors that facilitated cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system, version 2 (Amersham International) was used with oligo 1 (figure 4) to create FDTPs/Bs. The sequence of FDTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, USA.) with oligo 3 (figure 4) as a primer.

A second vector FDTPs/Xh (to facilitate cloning of single chain Fv fragments) was generated by mutagenising FDTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The

sequence of FDTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For example, M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified protein, could be incorporated into a plasmid containing a single stranded phage replication origin, such as pUC119, superinfection with modified phage such as K07 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as FDTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989 Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin Fv Domain into Phage Antibody

The plasmid scFv D1.3 myc (gift from G. Winter and A. Griffiths) contains V_H and V_L sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain Fv version of antibody D1.3. The sequence of the scFv and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E. coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and Xhol, excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into FDTPs/Xh cleaved with PstI

and Xhol gave rise to the construct FDTSCFVD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector FDTPs/Xh was prepared for ligation by digesting with the PstI and Xhol for 2 hours followed by digestion with calf intestinal alkaline phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a final total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes, extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1 except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above, and the equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM Tris, 380 mM Glycine, 0.1%SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

After transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Cadbury's Marvel) in phosphate buffered saline (PBS). Detection of Fv and VH protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal

antiserum raised against affinity purified, bacterially expressed F_v fragment (gift from G. Winter). After washing PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and developed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results show that with FDTVHD1.3 (from example 3) and FDTSCVFVD1.3, a protein of between 69,000 and 92,500 daltons is detected by the anti-F_v serum. This is the expected size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, FDT₆Bst or FDTPs/Xh.

Example 3.

Insertion of Immunoglobulin V_H Domain into Phage Antibody

The V_H fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion with PstI and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the PstI and BstEII sites of FDTPs/Bs gave rise to the construct FDTVHD1.3 which encodes a fusion protein with a complete V_H inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was FDTPs/Bs digested with PstI and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies were grown in E.coli and phage antibody particles were precipitated with PEG as in the materials and methods. Bound phage antibody particles were detected using polyclonal rabbit serum raised against the

closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, California, 93030, USA.) with 200 ul of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mM NaHCO₃ for 16-24 hours. Before use, this solution was removed, the plate rinsed several times in PBS and incubated with 200 ul of 2% milk powder/PBS for 1 hour. After rinsing several times with PBS, 100 ul of the test samples were added and incubated for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 ul/well of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plates were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS=2'2'-azinobis(3-ethylbenzthiazoline sulphonic acid); citrate buffer =50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46). Hydrogen peroxide was added to a final concentration of 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibody. 100 ul of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (FDTSCFVD1.3) and the V_H containing phage antibody (FDTVHD1.3) were higher than that derived from the phage antibody vector (FDTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from FDTSCFVD1.3 were again higher than those derived from FDTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme coated. These results demonstrate that the binding detected is specific for lysozyme as the antigen.

Example 5.

Construction of fd Cat 2

It would be useful to design vectors that enable the use of restriction enzymes that cut DNA infrequently, thus avoiding unwanted digestion of the antibody gene inserts within their coding sequence. Enzymes with an eight base recognition sequence are particularly useful in this respect, for example NotI and SfiI. Chaudhary et al (PNAS 87 p1066-1070, 1990) have identified a number of restriction sites which occur rarely in antibody variable genes. The applicant has designed and constructed a vector that utilises two of these sites, as an example of how this type of enzyme can be used. Essentially sites for the enzymes ApaI and NotI were engineered into FDTPs/Xh to create fdCAT2.

The oligonucleotide:

5'ACT TTC AAC AGT TTC TGC GGC CGC CCG TTT GAT CTC GAG CTC
CTG CAG TTG GAC CTG TGC ACT GTG AGA ATA GAA 3'

was synthesised (supra fig 4 legend) and used to mutagenise FDTPs/Xh using an in vitro mutagenesis kit from Amersham International as described in example 1, to create fd-CAT2. The sequence of fd-CAT2 was checked around the site of manipulation by DNA sequencing. The final sequence around the insertion point within gene III is shown in figure 8.

Example 6

Specific Binding of Phage-antibody(pAb) to Antigen

The binding of pAb D1.3 (FDTSCFVD1.3 of example 2) to lysozyme was further analysed by ELISA.

Methods.1. Phage growth.

Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x YT medium with 15 µg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was $1 - 5 \times 10^{10}$ ml⁻¹ transducing units. The phage were precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge.

ELISA

Plates were coated with antigen (1 mg ml⁻¹ antigen) and blocked as described in example 4. 2×10^{10} phage transducing units were added to the antigen coated plates in phosphate buffered saline (PBS) containing 2% skimmed milk powder (MPBS). Plates were washed between each step with three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase (HRP) conjugated anti-goat serum (Sigma, Poole, Dorset, UK) and ABTS (2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid). Readings were taken at 405 nm after a suitable period. The results (figure 9) show that the antibody bearing-phage had the same pattern of reactivity as the original D1.3 antibody (Harper, M., Lema, F., Boulot, G., and Poljak, F.J. (1987) Molec. Immunol. 24, 97-108), and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the

lack of binding to the turkey egg-white lysozyme that differs from hen egg-white lysozyme by only 7 amino acids.

Example 7

Expression of Fab D1.3 in fd CAT2

The aim of this example was to demonstrate that the scFv format used in example 2 was only one way of displaying antibody fragments in the pAb system. A more commonly used antibody fragment is the Fab fragment (figure 1) this example describes the construction of a pAb that expresses a Fab-like fragment on its surface and shows that it binds specifically to its antigen. The applicant chose to express the heavy chain of the antibody fragment consisting of the VH1 and CH1 domains in the pAb itself and to co-express the light chain in the same cell; the VH and CH1 regions of anti-lysozyme antibody D1.3 were cloned in fd CAT2, and the corresponding light chain cloned in plasmid pUC19. The light chain associates with the heavy chain-gene III fusion as it does when the heavy and light chains are expressed as independant proteins (Skerra, A and Pluckthun, A. Science 240, p1038-1040 (1988)).

It is possible to express the light chain from within the pAb genome by, for example, cloning an expression cassette into a suitable place in the phage genome. Such a suitable place would be the intergenic region which houses the multicloning sites engineered into derivative of the related phage M13 (see, for example, Yanisch-Perron, C. et al., Gene 33, p103-119, (1985)).

The starting point for this example was the clone Fab D1.3 in pUC19, a map of which is shown in figure 10. The sequence encoding the VH-CH1 region was PCR amplified from Fab D1.3 in pUC19 using oligonucleotides KSJ 6 and 7, which retain the Pst I site at the 5'end and introduce a Xho I site at the 3'end, to facilitate cloning into fd CAT2. The sequences for the oligonucleotides KSJ 6 and 7 are shown below.

KSJ6: 5' AGG TGC AGC TGC AGG AGT CAG G 3'

KSJ7: 5' GGT GAC CTC GAG TGA AGA TTT GGG CTC AAC TTT C 3'

PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled (see example 12). This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst 1/Xho 1-cut fd CAT2 as described in example 12. Part of the ligation was transformed into *E.coli* MC1061 (Available from, for example Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2: VHCH1 D1.3.

The heavy chain was deleted from fab D1.3 in pUC19 by Sph I cleavage of fab D1.3 plasmid DNA. The 2.7Kb fragment containing pUC19 and the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent *E.coli* TG1. Cells were plated on 2YT agar containing ampicillin (100 μ g/ml) and incubated at 30°C overnight. The resulting colonies were used to make miniprep DNA (Sambrook et al. supra), and the absence of the heavy chain gene confirmed by digestion with Sph I and Hind III. A representative clone was called LC D1.3 DHC.

An overnight culture of fd CAT2; VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50 μ l of the supernatant containing phage particles added to 50 μ l of an overnight culture of LC D1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2YT agar containing ampicillin (100 μ g/ml) and 15mg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6.

The results (Figure 11) showed that when the heavy and

light chain Fab derivatives from the original antibody D1.3 were present, the pAb bound to lysozyme. pAb expressing the fd VHCH1 fragment did not bind to lysozyme unless grown in cells also expressing the light chain. This shows that a functional Fab fragment was produced by an association of the free light chain with the VHCH1 fragment fused to gene III and expressed on the surface of the pAb.

Example 8

Isolation of Specific, Desired Phage from a Mixture of Vector Phage.

The applicant purified pAb (D1.3) (originally called FDTSCFVD1.3 in example 2) from mixtures using antigen affinity columns. pAb (D1.3) was mixed with vector fd phage (see table 1) and approximately 10^{12} phage passed over a column of lysozyme-Sepharose (prepared from cyanogen bromide activated sepharose 4B (Pharmacia, Milton Keynes, Bucks, UK.) according to the manufacturers instructions. TG1 cells were infected with appropriate dilutions of the elutes and colonies derived, were analysed by probing with an oligonucleotide that detects only the pAb (D1.3) (see Table 1 and Fig. 12) A thousand fold enrichment of pAb (D1.3) was seen with a single column pass. By growing the enriched phage and passing it down the column again, enrichments of up to a million fold were seen.

Enrichment was also demonstrated using purely immunological criteria. For example, 10^{12} phage (at a ratio of 1 pAb (D1.3) to 4×10^6 FDTPs/Bs was subjected to two rounds of affinity selection, and then 26 colonies picked and grown overnight. The phage was then assayed for lysozyme binding by ELISA (as example 6). Five colonies yielded phage with lysozyme binding activities, see table 1, and these were shown to encode the scFv (D1.3) by PCR screening (example 13, using 30 cycles of 1 minute at 92°C, 1 minute at 60°C, 1 minute at 72°C using CDR3PCR1 and oligo 3 (fig. 4) as primers).

Thus very rare pAbs can be fished out of large populations, by using antigen to select and then screen the phage.

Methods

Affinity Chromatography of pAbs

Approximately 10^{12} phage particles in 1ml MPBS were loaded onto a 1 ml lysozyme-Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris-HCl, 500 mM NaCl pH 7.5; then 10ml 50 mM Tris-HCl, 500 mM NaCl pH 8.5; then 5 mls 50 mM Tris-HCl, 500 mM NaCl pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralised with 0.5 M sodium phosphate buffer pH 6.8 and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per petri dish. After overnight growth, colonies were then scraped into 5 ml 2 x YT medium, and a 20 μ l aliquot diluted into 10 ml fresh medium and grown overnight. The phage was PEG precipitated as above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above.

Oligonucleotides synthesised:

CDR3PCR1 5'TGA GGA C(A or T) C(A or T)GC CGT CTA CTA CTG TGC 3'

Oligonucleotide probing

40 pmole oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 μ Ci γ -32P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x SSC buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC.

Example 9

Construction of pAb Expressing Anti-hapten Activity

Oxazalone is a hapten that is a commonly used for studying

the detail of the immune response. The anti-oxazalone antibody, NQ11 has been described previously (E. Gherardi, R. Pannell, C. Milstein, J. Immunol. Method 126 61-68). A plasmid containing the VH and VL genes of NQ11 was converted to a ScFv form by inserting the BstEII/SacI fragment of SCFVD1.3 myc (nucleotides 432-499 of Fig 5) between the VH and VL genes to generate pSCFVNQ11, the sequence of which is shown in fig 13. This ScFv was cloned into the PstI/Xhol site of FdTPs/Xh (as described earlier) to generate pAb NQ11. (NQ11 has an internal PstI site and so it was necessary to do a complete digest of pSCFVNQ11 with Xhol followed by a partial digest with PstI)

The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50mM NaHCO₃ at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazalone (OX-BSA) (method of conjugation in Makela O., Kaartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J.Exp.Med.148 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6: Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

Example 10

Enrichment of pAb D1.3 from Mixtures of Other pAb by Affinity Purification

3×10^{10} phage in 10mls of PBSM at the ratios of pAb D1.3 to pAb NQ11 shown in table 2 were passed over a 1ml lysozyme Sepharose column. Washing, elution and other methods were as described in example 8 unless otherwise stated. Elutes from the columns were used to infect TG1 cells which were

then plated out. Colonies were probed with a probe which distinguishes pAb D1.3 from pAb NQ11. The sequence of this oligonucleotide (D1.3CDR3A) is:-

5'GTA GTC AAG CCT ATA ATC TCT CTC 3'

Table 2 presents the data from this experiment. An enrichment of almost 1000 fold was achieved in one round and an enrichment of over a million fold in two rounds of purification. This parallels the result described in example 8.

Example 11

Insertion of the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB into fdCAT-2

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al., PNAS 85, p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primers also incorporate a unique ApaL1 site at the 5' end of the fragment and a unique Xhol site at the 3' end, to facilitate cloning into the vector fdCAT-2. The sequence of the primers is:

RPDGF1 5'C ACA GTG CAC GTC CTC AAT GTC TCC AGC ACC TTC 3'

RPDGF2 5'GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A., 1990 Nucl. Acids. Research 18 p3739-3744). The PCR mixture contained; 20mM Tris HCl (pH7.3 at 70°C), 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dGTP, dCTP, and dTTP, 500ng/ml RP41 DNA, 1μM each primer and 50 units/ml Tag polymerase (Cetus/Perkin-Elmer, Beaconsfield, Bucks, UK)

Thirty cycles of PCR were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 min and extension at 72°C for 3 min. This reaction resulted in amplification of a fragment of ca 1500bp as expected.

fdCAT-2 vector DNA was digested with ApaL1 and Xhol (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al., *supra*). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 12 except that probing was with 32-P labelled RPDGF1 and analytical PCR was performed using RPDGF1 and RPDGF2 as primers.

Example 12

Insertion of the Extracellular Domain of the Human Epidermal Growth Factor Receptor into fd-CAT2

A gene fragment encoding the extracellular domain of the human epidermal growth factor receptor (hEGF-R) was isolated by polymerase chain amplification of plasmid pJ3EGF-R (Clark et al. (1988) *J. Cell Physiol.* 134, p421-428) which contains the gene for hEGF-R (Ullrich, A. et al., *Nature* 309, p418-425, (1984)). The oligonucleotide primers used were designated to amplify the region of the hEGF-R gene corresponding to amino acid 1 of the mature protein through to amino acid 621. The primers also incorporate an unique ApaL1 site at the 5' end of the fragment and an unique Xho 1 site at the 3' end, to facilitate cloning into the vector fd-CAT2. The sequence of the primers is:

Oligo KSJ4: 5' GAT CTC GAG GGA CGG GAT CTT AAG CCC ATT CGT TGG 3'

Oligo KSJ5: 5' CAG AGT GCA CTG GAG GAA AAG AAA GTT TGC CA 3'
PCR amplification was carried out using high-fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 *Nucl. Acids Res.* 18, 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH 7.3 at 70°C), 50mM KCl, 4mM MgCl₂, 0.01%

gelatin, 1mM each of dATP, dGTP, dCTP and dTTP, 500ng/ml pJ3 plasmid, 0.5 μ M each primer and 50U/ml Taq polymerase (Cetus/Perkin-Elmer). Thirty cycles of PCR amplification were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes. This regime resulted in amplification of a fragment of the expected size (approximately 1800bp).

The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with ApaL 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations. The fragment was resolved on a 1% Tris-Acetate-EDTA agarose gel (Sambrook et al. supra.) and purified using Geneclean (BIO 101) (Geneclean, La Jolla, San Diego, California, USA) according to manufacturers reccomendations.

fd-CAT2 vector DNA was digested with ApaL 1 and Xho 1 (New England BioLabs) according to manufacturers reccomendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.).

75ng of ApaL 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified ApaL 1/Xho 1-digested hEGF-R fragment in 12 μ l of ligation buffer (66mM TrisHCl (pH7.6), 5mM MgCl₂, 5mM dithiothreitol, 100mg/ml bovine serum albumin, 0.5mM ATP, 0.5mM Spermidine) and 400 units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C.

Two μ l of the ligation mixture was transformed into 200 μ l of competent E.coli MC1061 cells, plated on 2YT agar containing 15mg/ml tetracycline and incubated at 30°C for 20 hours.

Colonies containing hEGF-R were identified by probing with ³²P-labelled KSJ 4 oligonucleotide (Sambrook et al. supra.) and the presence of an insert in hybridising colonies confirmed by PCR using the conditions described above. In this case the template DNA was prepared from the colonies by picking some colony material into 100 μ l of

distilled water and boiling for 10 minutes. 1 μ l of this mixture was used in a 20 μ l PCR.

Example 13.

Insertion of a Gene Encoding an Enzyme (Alkaline phosphatase) into fd-CAT2

As an example of the expression of a functional enzyme on the bacteriophage surface, the applicants have chosen bacterial alkaline phosphatase an enzyme that normally functions as a dimer (McCracken, S. and Meighen, E., J. Biol. Chem. 255, p2396-2404, (1980)). The oligonucleotides were designed to generate a PCR product with an Apa L1 site at the 5' end of phoA gene and a Not 1 site at its 3' end, thus facilitating cloning into fd-CAT 2 to create a gene III fusion protein. The oligonucleotides synthesised were:

phoA1:5' TAT TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG 3' and,

phoA2:5' ACA TGT ACA TGC GGC CGC TTT CAG CCC CAG AGC GGC TTT C3'

The sequence of the phoA gene is presented in Chang C. N. et al., Gene 44, p121-125 (1986).

The PCR reaction was carried out in 100 μ l containing 50mM KC1, 2.5mM MgCl₂, 0.01% gelatin, 10mM Tris/HCl pH 8.3, 0.25 units/ μ l of Taq polymerase (Cetus/Perkin Elmer) and 0.5 μ g/ml template. The template was the pEK plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C.

The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35 μ l water. Digestion with 0.3 units/ μ l of Apa L1 was carried out in 150 μ l volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C, NaCl was added to a final concentration of 150mM and 0.4 units/ μ l Not1 enzyme

added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30µl of water. The vector fd-CAT2 was sequentially digested with Apa L1 and Not1 according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/µl of both the cut fd-CAT2 and then digested PCR product. The ligations were transformed into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoA1. The sequence at the junction of the cloning region is given in figure 15.

Example 14

Measuring Enzyme Activity of Phage-enzyme

Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoA1 or fd-CAT2 were grown at 37°C in 2xTY with 15µg/ml tetracycline. Concentrated, PEG precipitated phage were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at 24°C in a final concentration of 1M Tris/HCl pH 8.0, 1mM nitrophenyl phosphate (Sigma), 1mM MgCl2. 100µl of a two times concentrate of this reaction mixture was mixed with 100µl of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000.

Standard curves (amount of enzyme vs. rate of change of absorbance) were prepared using dilutions of purified bacterial alkaline phosphatase (Sigma type III) in 10mM Tris/HCl pH 8.0, 1mM EDTA. The number of enzyme molecules in the phage samples were estimated from the actual rates of change of absorbance of the phage samples and comparison to this standard curve.

The results in Table 3 show that alkaline phosphatase activity was detected in PEG precipitated material in the sample containing fd-phoA1 but not fd-CAT2. Furthermore, the level of activity was consistent with the expected number of 1-2 dimer molecules of enzyme per phage. The level of enzyme activity detected was not dependant on the host used for growth. In particular, fd-phoA1 grown on phoA minus hosts showed alkaline phosphatase activity.

Therefore, the phage express active alkaline phosphatase enzyme from the phoA-gene III fusion on the phage surface.

Example 15

Insertion of Binding Molecules into Alternative Sites in the Phage

The availability of an alternative site in the phage for the insertion of binding molecules would open up the possibility of expressing more than one antibody fragment in a single pAb. This may be used to generate single or multiple binding specificities. The presence of two distinct binding activities on a single molecule will greatly increase the utility and specificity of this molecule. It may be useful in the binding of viruses with a high mutational rate such as human immunodeficiency virus. In addition, it may be used to bring antigens into close proximity (eg. drug targetting or cell fusion) or it may act as a "molecular clamp" in chemical, immunological or enzymatic processes.

The vector fd-tet and the derivatives described here, have a single BamH1 site in gene 3. This has previously

been used for the expression of peptide fragments on the surface of filamentous bacteriophage (Smith GP. (1985) *Science* 228 p1315-1317 and de la Cruz et al. (1988) *J Biol. Chem.* 263 p4318-4322). This provides a potential alternative site for the insertion of antibody fragments.

DNA fragments encoding SCFv's from D1.3 or NQ11 were generated by PCR using the primers shown below. These primers were designed to generate a fragment with BamH1 sites near both the termini, to enable cloning into the BamH1 site of gene3 (see figure 16(1)). The oligonucleotides used, also ensure that the resulting PCR product lacks Pst1 and Xhol restriction sites normally used for manipulating the SCFv's (see figure 16(1)). This will facilitate subsequent manipulation of a second antibody fragment in the usual way at the N terminus of gene 3. The oligonucleotides used were:-

G3Bam1 5' TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG 3'

G3Bam2 5' AAC GAA TGG ATC CCG TTT GAT CTC AAG CTT 3'.

Preparation of vector and PCR insert

The PCR reaction was carried out in an 80 μ l reaction as described in example 13, using 1ng/ μ l of template and 0.25U/ μ l of Taq polymerase and a cycle regime of 94°C for 1 minute, 60°C for 1 minute and 70°C for 2 minutes over 30 cycles. The template was either pSCFvNQ11 (example 9) or SCFvD1.3 myc (example 2). Reaction products were extracted with phenol:chloroform, precipitated, dissolved in water and digested with BamH1 according to manufacturers instructions. The digest was re-extracted with phenol:chloroform, precipitated and dissolved in water.

The vector FDTPs/Xh was cleaved with BamH1 and treated with calf intestinal phosphatase and purified as described in example 2. Ligations were set up at a vector concentration of approximately 6ng/ μ l and a PCR insert concentration of approximately 3ng/ μ l. These were ligated for 2.5 hours at room temperature before transforming into

competent TG1 cells and plating on TYE tet plates. The resultant colonies were probed as described in example 8. DNA was prepared from a number of colonies and the correct orientation and insert size confirmed by restriction digestion with Hind III in isolation or in combination with BamH1. (One Hind III site is contributed by one of the primers and the other by the vector).

Two clones containing a D1.3 insert (FDTBam1 and FDTBam2) and one containing an NQ11 insert (NQ11Bam1) were grown up and phage prepared as described earlier. ELISAs were carried out as described in example 6. No specific signal was found for any of these clones suggesting that the natural BamH1 site is not a suitable site for insertion of a functional antibody (results not shown).

It may be possible to clone into alternative sites to retain binding activity. The peptide repeats present in gene3 may provide such a site (figure 16 blocks A and B). This can be done by inserting a BamH1 site and using the PCR product described above. To facilitate this, the natural BamH1 site was removed by mutagenesis with the oligonucleotide shown below (using an in vitro mutagenesis kit (Amersham International)):-

G3mut~~B~~Bam 5' CA AAC GAA TGG GTC CTC CTC ATT A 3'

The underlined residue replaces an A residue thereby removing the BamH1 site. DNA was prepared from a number of clones and several mutants lacking BamH1 sites identified by restriction digestion.

The oligonucleotide G3 Bamlink was designed to introduce a BamH1 site at a number of possible sites within the peptide linker sites A and B, see figure 16(2). The sequence of the linker is:

5'CC (G or A) CC ACC CTC GGA TCC (G or A) CC ACC CTC 3'

Its relationship to the peptide repeats in gene III is shown in figure 16.

Table 1. Enrichment of pAb (D1.3) from vector population

INPUT RATIO ^a pAb : fd-CAT1	OUTPUT RATIO		ENRICHMENT ^d
	oligo ^b	ELISA ^c	
Single Round			
1 : 4x10 ³	43/124		1.3 x 10 ³
1 : 4x10 ⁴	2/82		1.0 x 10 ³
Two Rounds			
1 : 4x10 ⁴	197/372		2.1 x 10 ⁴
1 : 4x10 ⁵	90/356	3/24	1.0 x 10 ⁵
1 : 4x10 ⁶	27/183	5/26	5.9 x 10 ⁵
1 : 4x10 ⁷	13/278		1.8 x 10 ⁶

Footnotes: ^aApproximately 10¹² phage with the stated ratio of pAb (D1.3) : FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted. ^bTG1 cells were infected with the eluted specific binding phage and plated onto TY-ctt plates. After incubation overnight at 30-37°C the plates were analysed by hybridisation to the ³²P-labelled oligonucleotide VH1FOR (Ward et al op cit) which is specific to pAb D1.3. ^cSingle colonies from overnight plates were grown, phage purified, and tested for lysozyme binding. ^dEnrichment was calculated from the oligonucleotide probing data.

Table 2 Enrichment of pAb(D1.3) from mixed pAb population

Input Ratio ¹ (pAbD1.3:pAbNQ11)	Output Ratio ² (pAb D1.3: Total phage)	Enrichment
Single Round		
1 : 2.5 x 10 ⁴	18/460	0.98 x 10 ³
1 : 2.5 x 10 ⁵	3/770	0.97 x 10 ³
1 : 2.5 x 10 ⁶	0/112	
pAb NQ11 only	0/460	
Second Round		
1 : 2.5 x 10 ⁴	119/170	1.75 x 10 ⁴
1 : 2.5 x 10 ⁵	101/130	1.95 x 10 ⁵
1 : 2.5 x 10 ⁶	102/204	1.26 x 10 ⁶
1 : 2.5 x 10 ⁷	0/274	
1 : 2.5 x 10 ⁸	0/209	
pAb NQ11 only	0/170	

Notes

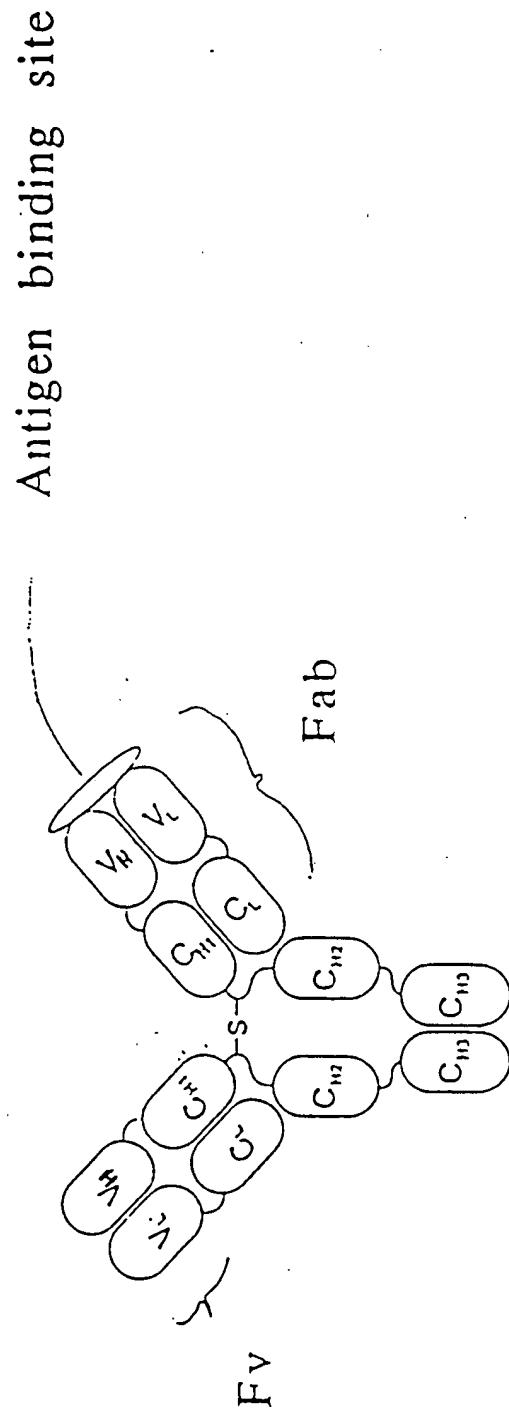
1. 10¹⁰ phage applied to a lysozyme column as in table 1.

2. Plating of cells and probing with oligonucleotide as in table 1, except the oligonucleotide was D1.3CDR3A

Table 3: Enzymic activity of phage-enzyme

Input	ng of enzyme or No. of phage	Rate (OD/hr)	No. of molecules of Enzyme equivalent ($\times 10^{-11}$)
Pure Enzyme	335	34	24.5
Pure Enzyme	177.5	17.4	12.25
Pure Enzyme	88.7	8.7	6.125
Pure Enzyme	44.4	4.12	3.06
Pure Enzyme	22.2	1.8	1.5
Pure Enzyme	11.1	0.86	0.76
No Enzyme	0	0.005	0
fd-phoA1/TG1	1.83×10^{11}	5.82	4.2
fd-CAT2/TG1	1.0×10^{12}	0.155	0.112
fd-phoA1/KS272	7.1×10^{10}	10.32	7.35
fd-CAT2/KS272	8.2×10^{12}	0.038	0.027

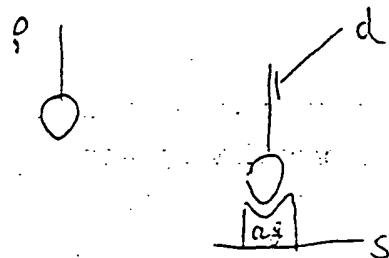
FIGURE 1: Antibody structure



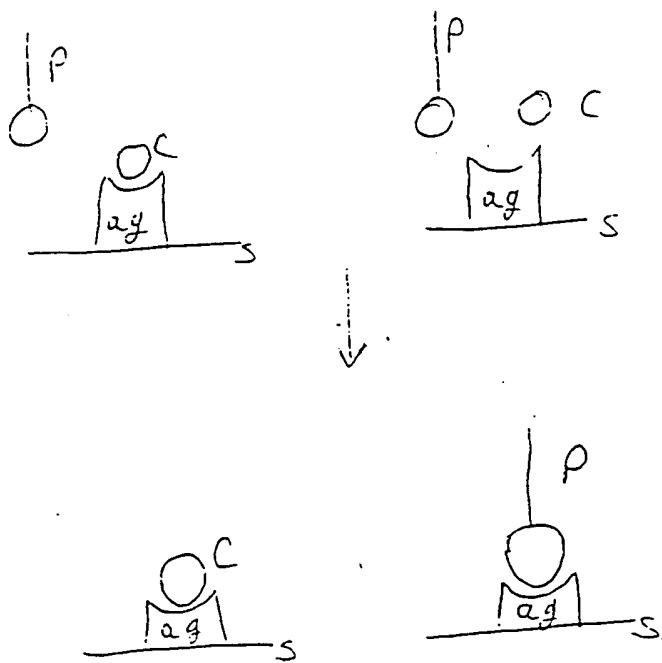
The single domain antibody (dAb, Ward et al. 1989) consists of a single V_H domain.

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FIGURE 2: ASSAY FORMATS

2 i) Binding/elution



2 ii) Competition



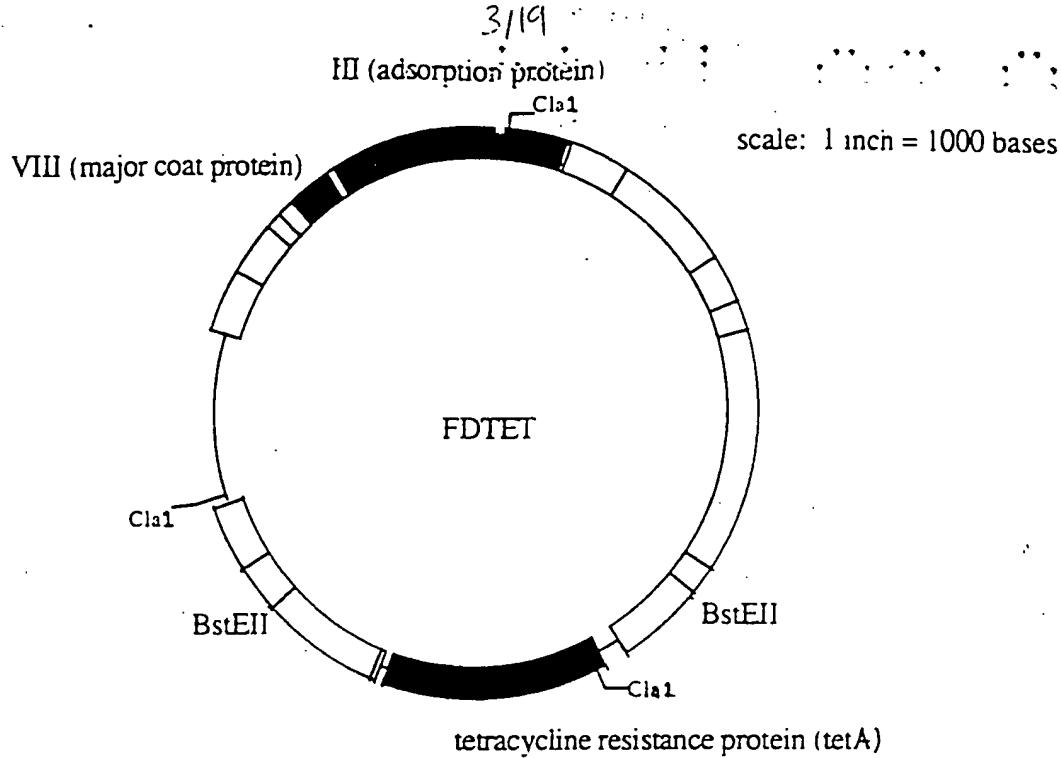
p - Phage antibody population to be sampled.

ag - Antigen to which binding required.

c - Competitor antibody/ phAb/ligand etc population.

s - Surface (eg plastic, beads etc).

d - Detection system



fd-tet

▼

cleave with BstEII

▼

fill in with Klenow

▼

re-ligate

▼

FDT δ Bst

▼

in vitro mutagenesis (oligo1)

▼

FDTPs/Bs

▼

in vitro mutagenesis (oligo 2)

▼

FDTPs/Xh

Figure 3 Scheme for construction of vectors

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1

(1853)

Oligo 1 ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC
GGA GTG AGA ATA (1620)

(1853)

Oligo 2 ACA AC1 TTC AAC AGT T1C CCG T11 GA1 CTC GAG CTC CTG CAG T1G GAC CTG

(1704)

Oligo 3 GTC GTC TTT CCA GAC GTT AGT

2

GENE III

GENE III

SIGNAL

CLEAVAGE SITE

(1624)

A TCT CAC TCC GCT

(1650)

GAA ACT GTT GAA AGT

Q V Q L Q V T V S S

B TCT CAC TCC GCT CAG GTC CAA CTG CAG AAG CTT ACG GTC ACC GTC TCC TCA ACT GTT GAA AGT
PstII BstEII

Q V Q L Q L E I K R

C TCT CAC TCC GCT CAG GTC CAA CTG CAG GAG GTC GAG ATC AAA CGG GAA ACT GTT GAA AC
PstII XbaI

B = FDTPS / Bs

C = FDTPS / XbaI

Figure 4. Sequence of oligos and vectors

Figure 5. Sequence of SCFvD1.3myc

rbs M V V L I P T A E
 GCATGCAATTCTATTCAAGGAGACAGTCATAATGAAATACCTATTGCCCTACGGCAGCC

Sph1 Pab1B leader

PE18 Leader
A G I L L L A A Q P A M A Q V Q L Q E S
GCTGGATTGTTATTACTCGCTGCCAACCCAGCGATGGCCCAAGGTGGCAGCTGCAAGGAGTC

G P G L V A P S Q S L S I T C T V S G F
GGACCTGGCTGGTGGAGGCCATCACAGAGCTGTCCATCACATGACCGCTTCAGGGTTC

S L T G Y G V N W V R Q P P G K G L E W
TCCTTTAACCGCTATGGTGTAACTGGGTTGCCAGGCCAGGAAAGGGCTGGAGTCG

VED1.3
L G M I W G D G N T D Y N S A L K S R L
CTGGAAATGATTGGGGTGTGAAACAGACTATAATTCTAGCTCTCAAATCCAGACTG
252 262 272 282 292 302

S I S K D N S K S Q V F I L K M N S L H T
ACCATCAGCAAGGACAACTCCAGAGCCAGTTTTCTTAAGATGAACTAGCTGACACTT
312 322 332 342 352 362

S S G S D I E L T Q S P A S L S A S V G
 cgtggggggatcgGACATCGAGGTCACTCGATCTCCGGCTCCCTTCTGCTTGTGGAA
 490 500 510 520 530 540
 SacI

```

S T V T I T C R A S G N I H N Y L A W Y T
GAACTGTCACCATCACATGTCGAGCACTGGGAATATTCAACATTATTCAGCTATGGTAT
550 560 570 580 590 600

```

Q Q K Q G K S P Q I L V Y Y T T T L A D
CAGCAGAAACAGGGAAAATCTCTCTACGCTCTCTGGCTATTATACAAACCTTACGGAT
610 620 630 640 650 660

VRD1.3

G	V	P	S	R	F	S	G	S	G	S	G	T	Q	Y	S	L	K	I	N
GGTGTCCTCATCAAGGTCAGTGGCAGTCAGGAACTCAAAATTATTCCTCAAGATCAAC																			
672	680	688	696	704	712	720	728	736	744	752	760	768	776	784	792	800	808	816	824

Myc Tag (TAG1)

T F G G G T K L E I Z R E Q V I I S F F
 ACGTTTCGTTGGGGCACCAAGCTTCTGATCTAAACGGAAACAAAACCTCTATCTCAGAGAG
 790 . 800 810 820 830 840
 XbaI

GATCTGAATTATAATGATCAAAAGGTTAATAGGATCCAGTCAGTCAGT
856 868 876 885

EcoB-
-

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Binding of phage antibodies to lysozyme:
varying amount of supernatant

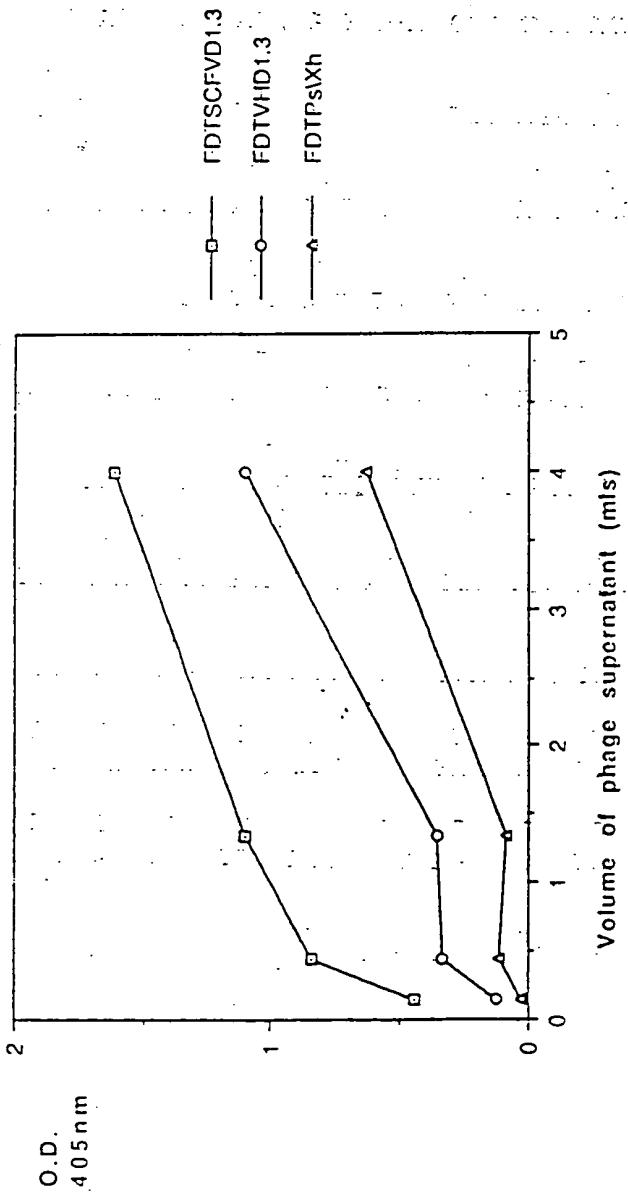
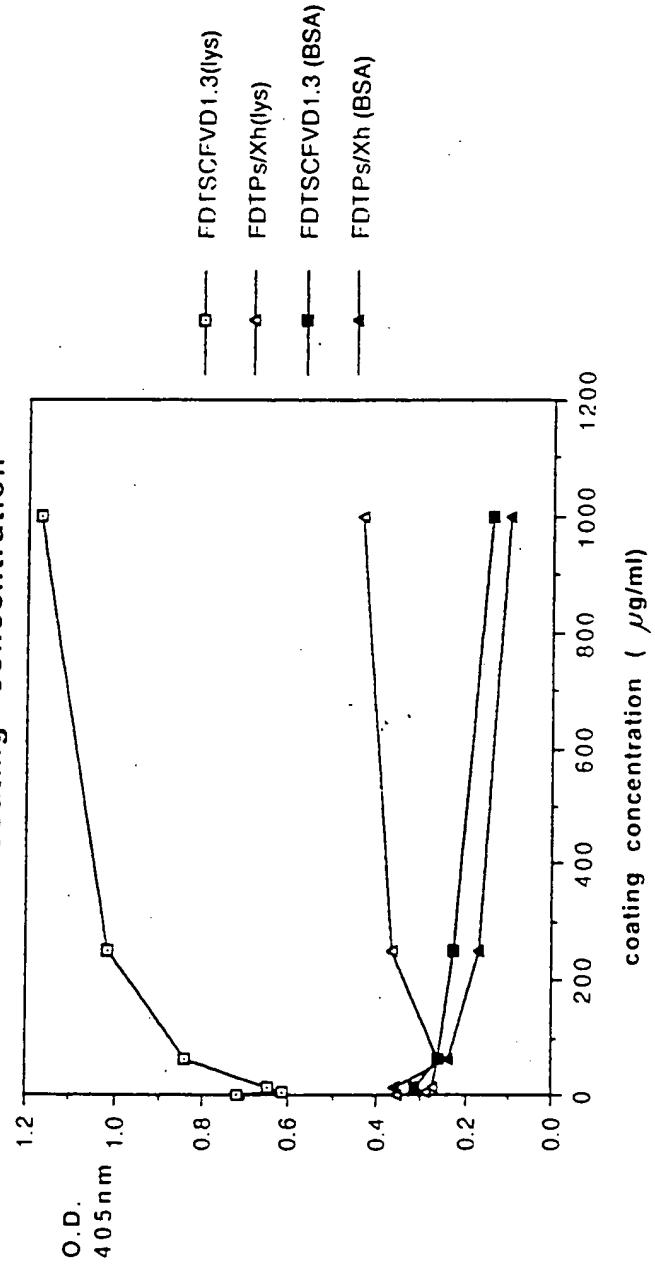


Fig. 6.

Methods as described in example 4

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Fig. 7

Binding of phage antibodies to lysozyme:
effect of coating concentration



Methods as described in example 4

8:19:

Fig. 8. Sequence around the cloning site of fd-CAT2.

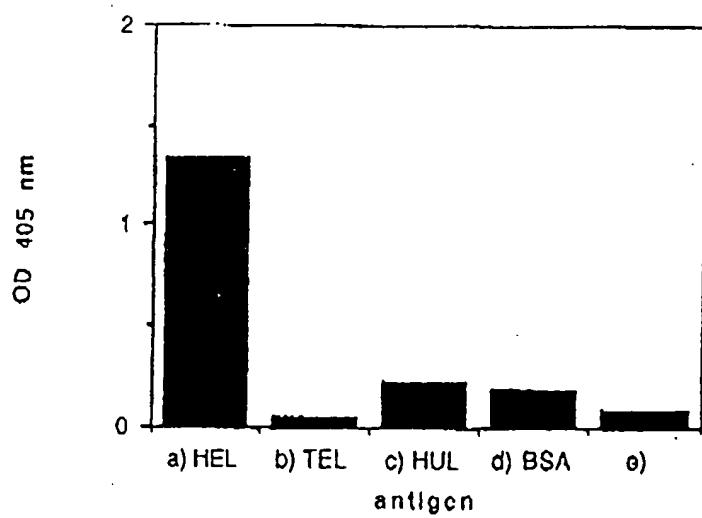
Cloning sites: Q V Q L Q L E I K R
 TAT TCT CAC AGT GCA CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG
 ↓
 Apa LI Pst I Xba I

GCG GCC GCA GAA ACT GTT GAA AGT etc.
Not I

Restriction enzyme sites are shown as well as the amino acids encoded by antibody derived sequences. These are flanked at the 5' end by the gene 3 signal peptide and at the 3' end by 3 alanine residues (encoded by the Not 1 restriction site) and the remainder of the mature gene 3 protein.

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Figure 9 Binding of pAb(D1.3) to lysozymes



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Figure 10: Sequence of Fab D1.3

M K Y L L P T A A A A G L L L P A A A Q P A
CATGCAAATTCTATTCAGGAGACAGTCATAATGAAATACCTATTCGCTACGGCAGCCGCTGGATTGGTATTACCTGCTGCCAACAG
11 21 31 41 51 61 71 81 91

M A Q V Q L Q E S G P G L V A P S Q S L S I T C T V S G F S
CGATGGCCCAAGGTGCAGCTGCAGGAGTCAGGACCTGGCTGGCTGGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCT
101 111 121 131 141 151 161 171 181

L T G Y G V N W V R Q P P G K G L E W L G M I W G D G N T D
CAITTAACCGGCTATGGGTAAACTGGGTTGCCAGCCTCCAGGAAAGGGCTGGAGTGGCTGGGAATGATTGGGTGATGGAAACACAG
191 201 211 221 231 241 251 261 271

Y N S A L K S R L S I S K D N S K S Q V F L K M N S L H T D
ACTATAATTCACTCTCAAATCCAGACTGAGCATCAGCAAGGACAACCTCCAAGAGCCAAGTTTCTTAAATGAACAGTCTGCACACTG
281 291 301 311 321 331 341 351 361

D T A R Y Y C A R E R D Y R L D Y W G Q G T T V T V S S A S
ATGACACAGCCAGGTACTACTGIGCCAGAGAGAGATTATAGGCTTGACTACTGGGCAAGGCACCCGGTACCGTCTCCCTCAGCCT
371 381 391 401 411 421 431 441 451

T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y
CCACCAAGGGCCCATGGTCTTCCCCCTGGCACCCCTCCAAGAGCACCTCTGGGGCACAGGGCCCTGGCTGCCCTGGTCAAGGACT
461 471 481 491 501 511 521 531 541

F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L
ACTTCCCCGAACGGTGACGGTGTGGAACTCAGGCGCCCTGACCAGGGCGTGCACACCTTCCCCCTGCTACAGTCTCAGGAC
551 561 571 581 591 601 611 621 631

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Fig. 10. cont. (1)

S L S S V V T V P S S S L G T Q T Y I C N V N H N P S N T
TACTCCCTCAGCAGCGTGGTGAATGTGCCCTCCAGCAGCTGGGACCCAGACCTACATCTGCAACGTGAATCACAACCCAGCAACA
641. 651 661 671 681 691 701 711 721

K V D K K V E P K S S * * * M K
CCAAGGTCGACAAGAAAGTTGAGCCAAATCTCATAATAACCCGGAGCTTGCATGCAAATTCTATTTCAGGAGACAGTCATAATGAA
731 741 751 761 771 781 791 801 811

L L P T A A A G L L L P A A A Q P A M A D I E L T Q S P A S
ATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCAGCCTC
821 831 841 851 861 871 881 891 901

L S A S V G E T V T I T C R A S G N I H N Y L A W Y Q Q K Q
CCTTCTGCGCTGTGGGAGAAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTACAATTATTAGCATGGTATCAGCAGAAACA
911 921 931 941 951 961 971 981 991

G K S P Q L L V Y Y T T T L A D G V P S R F S G S G S G T Q
GGGAAAATCTCCTCAGCTCCIGGCTATTATAACAACCTTAGCAGATGGTGTGCCATCAAGGTTAGTGGCAGTGGATCAGGAACACA
1001 1011 1021 1031 1041 1051 1061 1071 1081

D

Y S L K I N S L Q P E D F G S Y Y C Q H F W S T P R T F G G
ATATTCTCTCAAGATCAACAGCCTGCAAGATTGGAGATATTACTGTCACATTTGGAGTACTCCTCGGACGTTGGTGG
1091 1101 1111 1121 1131 1141 1151 1161 1171

G T K L E I K R T V A A P S V F I F P P S D E Q L K S G T A
AGGCACCAAGCTCGAGATCAAACGGACTGIGGCTGCACCATCTGCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACCTGC
1181 1191 1201 1211 1221 1231 1241 1251 1261

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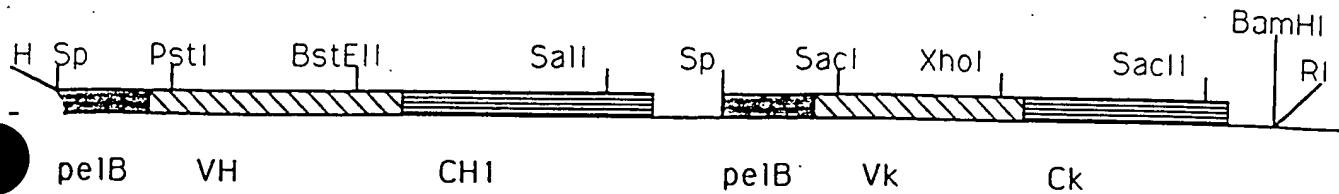
Fig. 10. cont. (2)

V V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q
CTCTGTTGTTGCTGCTGAATAACTCTATCCCAGAGAGGCCAAAGTACAGTGGAAAGGTGGATAACGCCCTCCAATGGGTAACCTCCA
1271 1281 1291 1301 1311 1321 1331 1341 1351

E S V T E Q D S K D S T Y S L S S T L T L S K A D Y E K H K
GGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGAAGCTGAGCAAAGCAGACTACGAGAAACACAA
1361 1371 1381 1391 1401 1411 1421 1431 1441

V Y A C E V T H Q G L S S P V T K S F N R G E S * *
AGTCTACGCCCTGCGAAGTCACCCATCAGGGCTGAGTTGCCCCGTACAAAGAGCTCAACCGCGGAGAGTCATAGTAAGGATCCAGCT
1451 1461 1471 1481 1491 1501 1511 1521 1531

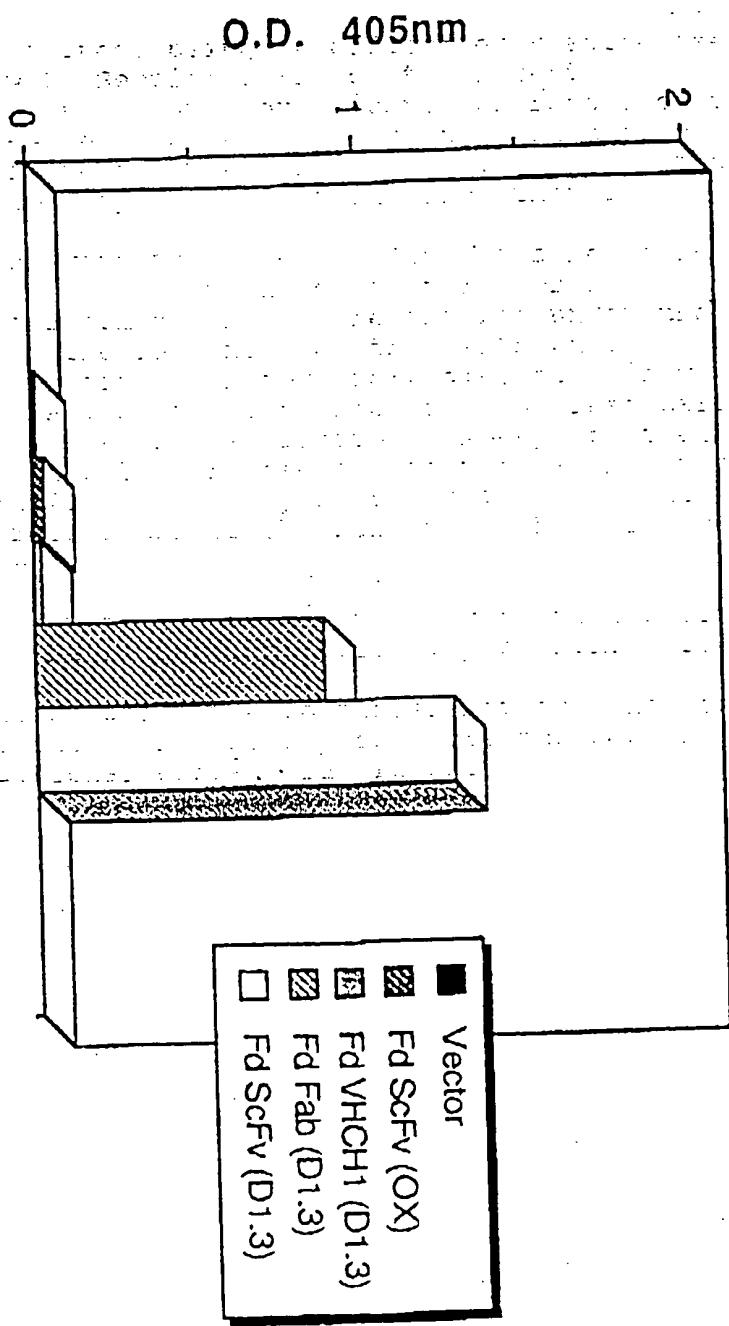
GAATTC



FabD1.3 in
pUC19

Fig. 10 cont. (3)

Fig. 11 Comparison of lysozyme-binding by phage-Fab and phage-ScFv



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Fig. 12

B

A

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Figure 13 Sequence of pNQ11ScFv

Q V Q L O E S G G G L V Q P G G
 CAG GTG CAG CTG CAG GAG TCA GGA GGA GGC TTG GTA CAG CCT GGG GGT
 PstI
 S L R L S C A T S G F T F S N Y
 TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT AAT TAC
 Y M G W V R Q P P G K A L E W L
 TAC ATG GGC TGG GTC CGC CAG CCT CCA GGA AAG GCA CTT GAG TGG TTG
 G S V R N K V N G Y T T E Y S A
 GGT TCT GTT AGA AAC AAA GTT AAT GGT TAC ACA ACA GAG TAC AGT GCA
 S V K G R F T I S R D N F Q S I
 TCT GTG AAG GGG CGG TTC ACC ATC TCC AGA GAT AAT TTC CAA AGC ATC
 L Y L Q I N T L R T E D S A T Y
 CTC TAT CTT CAA ATA AAC ACC CTG AGA ACT GAG GAC AGT GCC ACT TAT
 Y C A R G Y D Y G A W F A Y W G
 TAC TGT GCA AGA GGC TAT GAT TAC GGG GCC TGG TTT GCT TAC TGG GGC
 Q G T L V T v s s g g g g s g g g g s
 CAA GGG ACC CTG GTC ACC gtc tcc tca ggtggaggcggttcaggcggaggtggctct
 BstEII
 g g g g s d i E L T Q T P L S L P V
 ggcgggtggcggtatcgac atc GAG CTC ACC CAA ACT CCA CTC TCC CTG CCT GTC
 SacI
 S L G D Q A S I S C R S S Q S I
 AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT
 V H S N G N T Y L E W Y L Q K P
 GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
 PstI
 G Q S P K L L I Y K V S N R F S
 GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT
 G V P D R F S G S G S G T D F T
 GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCG GGG ACA GAT TTC ACA
 L K I S R V E A E D L G V Y Y C
 CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC
 F Q G S H V P Y T F G G G T K L
 TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTC
 E I K R
 GAG ATC AAA CGG
 XbaI

Restriction sites referred to in the text are shown underlined. The sequence contributed by the linker is shown in lower case.

Figure 14. Binding of pAbs to specific antigens

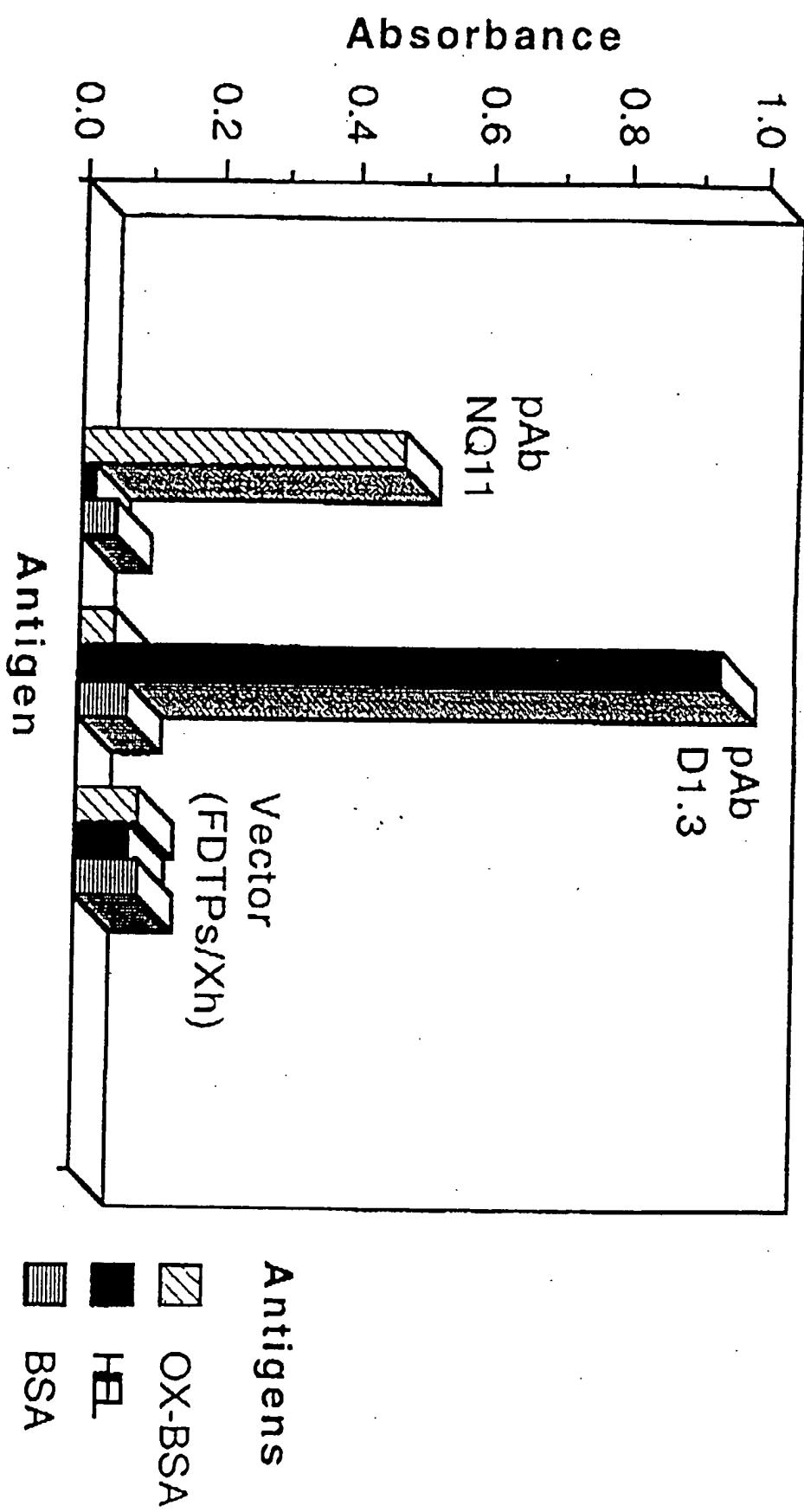


Fig.15. Sequence surrounding phoA insertion in fd-phoA1

SEQUENCE AT 5' END OF phoA INSERTION IN fd-phoA1

Signal peptide
cleavage site
▼ R T P E M P V L
TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG
ApaL1

SEQUENCE AT 3' END OF phoA INSERTION IN fd-phoA1

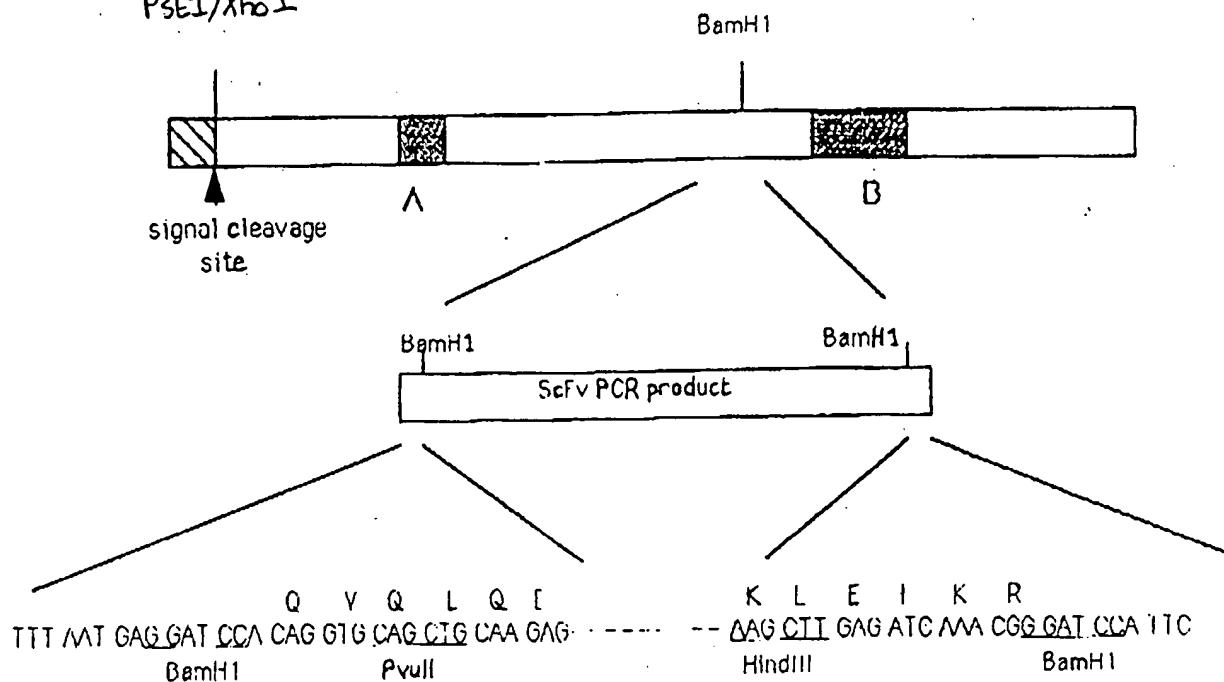
K A A L G L K
AAA GCC GCT CTG GGG CTG AAA GCG GCC GCA GAA ACT GTT GAA AGT etc.
NotI

The restriction sites used for cloning are shown as well as the amino acids encoded by phoA around the insertion site. In this example, the first five amino acids of the mature fusion will actually come from gene 3.

Figure 16. Structure of gene 3

1)

Pst1/Xba1



2)

A

(1834) 5' GAG GGT GGT GGC TGT
" " "C " "
" " "C " "
" " "C " ACT 3' (1839)

B

(2284) 5' - GGC GGC GGC TCT
 . GGT CGT GGT "
 . " GGC GGC "
 GAG " " GGC "
 " " " GGT "
 " " " GGC "
 " " " GGT "
 " " " GGC " 3' (2379)

Reverse complement of mutagenic
oligo G3 β am1ink

5' GAG GGT CGC GGA TCC 3'

1
GAG GGI GGC GG 3'

Numbering is according to Beck et al (1978, *supra*).

PCT/GB/91/01134

18.7.91

Memorandum

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